In Vivo β-Adrenergic Stimulation Suppresses Natural Killer Activity and Compromises Resistance to Tumor Metastasis in Rats[1]

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The sympathetic nervous system has been implicated in mediating stress-induced alterations in NK cell activity, particularly through stimulation of β-adrenergic receptors. However, because catecholamines induce time-dependent alterations in the distribution of NK cells, the impact of β-adrenergic stimulation on individual NK cell cytotoxicity is not clear, nor are its implications regarding host resistance to metastatic spread. To address these issues, we used the β-adrenergic agonist, metaproterenol (MP), in F344 rats. The number of blood NK cells doubled within 10 min of MP administration and returned to baseline levels within 1 h. By this time, MP suppressed blood NK activity in a dose-dependent manner. Two β-adrenergic antagonists, propranolol, which crosses the blood-brain barrier, and nadolol, which does not, blocked this suppression. Corresponding findings were obtained using an NK-sensitive tumor model, the MADB106. MP caused an up to 10 times increase in the number of tumor cells retained in the lungs 1 day after inoculation and a similar rise in the number of consequent lung metastases detected 3 wk later. These effects were dose dependent and nadolol reversible. NK cells appear to play a central role in mediating the tumor-enhancing effects of MP because their selective depletion nearly abolished this effect. Overall, our findings suggest that independent of the transitory increase in numbers of blood NK cells, in vivo β-adrenergic stimulation suppresses NK activity in the rat. This suppression is induced peripherally and can compromise host resistance to NK-sensitive tumors. Homologies to studies in humans and clinical relevance are discussed. *The Journal of Immunology,* **1998, 160: 3251–3258.**

NK cells are believed to play a significant role in cellular resistance to viral disease (1) and malignancy (2), especially during metastatic formation (3, 4). Stress is known to modulate NK function. Under certain conditions, stress was shown to suppress NK activity in humans and animals (5). Animal studies also indicate that this suppression may compromise host resistance to tumor development and metastasis (6–8).

The extent of adrenergic involvement in the stress-induced alteration of NK activity in humans is unsettled; human NK cells express β-adrenergic receptors (9, 10), and in vitro studies have demonstrated suppression of NK activity at physiologic concentrations of adrenaline (as low as 10^{-11} M). This suppression was blocked by selective β or β_2 receptor antagonists (11–13). On the other hand, most of the studies assessing human NK activity following catecholamine administration, acute stress, or exercise reported an elevation in NK activity (14–19). This apparent inconsistency would be resolved provided this elevation reflects an increased proportion of NK cells within the cell population tested for NK activity (usually PBMCs) rather than an actual increase in individual NK cell cytotoxicity. It is now clear that stress causes a sharp increase in the proportion of NK cells within the circulating leukocytes (up to 600%). This increase is mediated by catecholamines (20–22), most likely through a reduction in NK cell adherence to endothelial cells (23) combined with an increase in blood flow (24). Studies attempting to compensate for the elevated numbers of NK cells (induced by stress or catecholamines) have yielded inconsistent findings; some reported a decrease in NK activity (25, 26), some reported no change (21), and others reported an increase (17, 27). The findings are further complicated by the time course of these effects. The rise in NK cell number is transitory, typically lasting up to 1 h; following this period, there are indications of suppressed NK activity (17, 26, 28) that may be unrelated to changes in NK cell numbers (26).

Although to the best of our knowledge, animal studies have not reported the impact of systemic administration of catecholamines on NK activity, they have provided abundant indirect evidence of sympathetic nervous system involvement in the suppression of NK activity. For example, rats and mice either treated s.c. with ethanol or neuroleptics or injected intracerebroventrically with IL-1 or corticotropin-releasing factor exhibited suppressed NK activity that could be attenuated by peripheral adrenergic blockade (29–32). It has also been suggested that sympathetic innervation of the spleen modulates immune function (33). Indeed, studies in rats have indicated that electrical stimulation of the splenic nerve suppressed splenic NK activity through β-adrenergic mechanisms (34), and that chemical sympathectomy modulated splenic NK activity (35). Finally, we have demonstrated suppression of host resistance to an NK-sensitive tumor in the rat following forced swimming (36) or social confrontation (37). These effects were partially blocked by β-antagonists and were abolished by adrenal demedullation. However, most studies in animals have assessed NK activity in the spleen and have not compensated for catecholamine-induced redistribution of NK cells. The implication of these findings in the inconsistency in the human literature is therefore unclear.

Apart from the uncertainty of whether adrenaline increases or decreases human NK activity in vivo and whether these changes

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are per NK cell, it is still unclear whether adrenergic activation can alter host resistance to tumor development by changing NK activity. This may be especially important when surgical removal of malignancies induces sympathetic activation under conditions of high metastatic risk.

Thus, the present study attempts to determine the impact of β-adrenergic stimulation on both the number and activity of NK cells, understand the mechanisms underlying this impact, and assess its biologic significance using an NK-sensitive tumor model. To this end, we studied the effect of systemic administration of β-adrenergic agonists and antagonists in F344 rats using two different approaches: ex vivo studies in which we assessed the number and activity of blood NK cells following drug administration, and in vivo studies in which we assessed either the number of tumor cells retained in the lung 24 h following i.v. inoculation or the number of consequent lung metastases visible weeks later. The tumor line used, MADB106, is syngeneic to the F344 rat, metastasizes only to the lungs, and is highly sensitive to NK activity in vivo (38, 39).

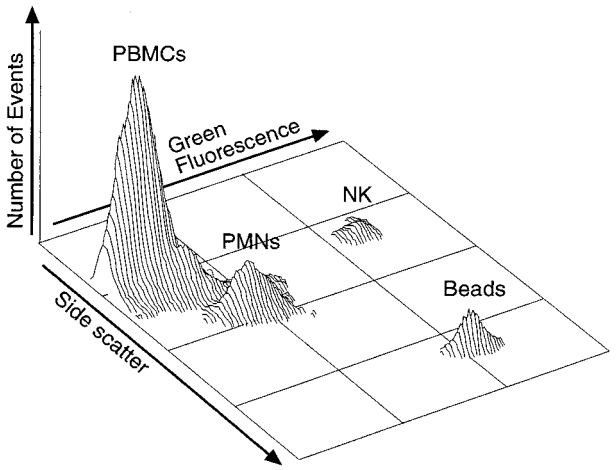

FIGURE 1. Analysis of different WBC populations based on green fluorescence (binding to anti-NKR-P1) and side scatter (granularity). Beads, plastic microbeads used as reference for total WBC number.

Materials and Methods

Animals

Fischer F344 male rats were purchased from Harlen Laboratories (Jerusalem, Israel) and housed four per cage in a reversed 12-h light/12-h dark cycle with free access to food and water. Animals were acclimatized to the vivarium for at least 4 wk before experimentation and were 14 to 20 wk old at that time. In any given experiment, all animals were of the same age.

Experimental design

To minimize the stress of injection, all rats were handled daily for 3 days before the experiments. The order of blood withdrawal, drug administration, and tumor injection was counterbalanced across groups in each experiment, and control animals were injected with saline. The numbers of animals used in Expt. 1 to 6 were 130, 95, 45, 66, 194, and 120, respectively. In Expt. 1, 2, 5, and 6, large numbers of animals were needed to obtain complete time-course or dose-response curves. These studies were conducted over two or more sessions, and their results were combined based on control levels from the different sessions. In each experiment, blood withdrawal or tumor inoculation was completed within 1 h and was conducted during the first half of the dark phase.

Materials

Metaproterenol (MP[3]). MP (Sigma, Holon, Israel) is a nonselective β-adrenergic agonist, with a higher affinity to β_2 receptors than to β_1 and a half-life of about 2 h in rats (40, 41).

Propranolol. Propranolol (Sigma) is a lipophilic nonselective β-adrenergic antagonist (42).

Nadolol. Nadolol (Sigma) is a hydrophilic analogue of propranolol and a nonselective β-adrenergic antagonist (42).

Anti-NKR-P1. Anti-NKR-P1 (PharMingen, San Diego, CA) is an mAb (originally termed mAb 3.2.3) that binds to a surface Ag (NKR-P1) expressed on fresh and IL-2-activated NK cells in the rat and, to a much lesser degree, on polymorphonuclear cells (PMNs) (43). In vivo treatment of rats with anti-NKR-P1 selectively depletes NK cells and eliminates NK- and Ab-dependent non-MHC-restricted cell cytotoxicity. T cell function and the percentages of T cells, PBMCs, and PMNs are unaffected (44, 45). Conjugated with FITC, this mAb was used in FACS analysis to identify NK cells.

Flow cytometry

An aliquot of 100 μl of blood was combined with 50 μl of PBS (supplemented with 2% FCS and 0.1% NaN_3) and 0.1 μg of FITC-conjugated anti-NKR-P1. Samples were subsequently kept in the dark at room temperature for 15 min before 2 ml of FACS lysis solution (Becton Dickinson, San Jose, CA) was added. Ten minutes later, samples were centrifuged for 5 min at 500 × *g*, and the lysis solution was aspirated. Cells were washed again with 2 ml of PBS and resuspended in 500 μl of PBS for flow cytometric analysis using a FACScan (Becton Dickinson). To assess the number of NK cells per microliter of blood, the suspending solution was supplemented with 300 polystyrene microbeads (20 μm in diameter; Duke Scientific, Palo Alto, CA) for each microliter of the original blood sample analyzed. The number of NK cells (NKR-P1^{bright}) per microliter of blood was calculated using the formula: NK/μl = (nk × 300)/beads, where nk and beads are the number of events associated with NKR-P1^{bright} cells and polystyrene microbeads, respectively. Applying this method repeatedly to the same blood sample in our laboratory yielded cell counts with an SD of 6%. No overlap between the bright and the dim cell populations was evident (Fig. 1). PBMCs and PMNs were identified by scatter properties and their differential binding to anti-NKR-P1. Because PMNs, but not PBMCs, are weakly labeled by the anti-NKR-P1 (43), using these properties allows better separation of cell populations than using scatter properties alone.

Whole blood NK activity assay (ex vivo studies)

This 4-h procedure assesses antitumor cytotoxicity in blood samples without separating PBMCs from other cells, minimizes the time between blood withdrawal and assessment of cytotoxicity, and reduces the interference with NK cell function. Our previous studies (46, 47) indicate that cytotoxicity in this assay depends on NK cells, since their selective depletion nullified all specific killing.

Rats were lightly anesthetized with halothane, and blood was drawn into a heparinized syringe (25 U/ml blood) by cardiac puncture. Exactly 1 ml of blood was washed once with PBS (diluted 1/3 (v/v), centrifuged at 300 × *g* for 10 min, and supernatant aspirated to original blood volume) and twice with complete medium (RPMI 1640 medium (Biologic Industries, Beit Haemek, Israel) supplemented with 10% heat-inactivated FCS, 50 μg/ml gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate). For each of the six E:T cell ratios used, 100 μl of washed blood was placed into each well of a microtiter plate, and 150 μl of ^{51}Cr-labeled YAC-1 tumor cells in complete medium was added on top of the blood. A concentration of 1.6×10^6/ml YAC-1 was used for the lowest E:T cell ratio (~1:8 NK:YAC-1, depending on individual NK concentration) and sequentially diluted by 2 to produce higher E:T cell ratios (~4:1 at the highest). Spontaneous (SP) and maximal (MAX) release of ^{51}Cr from target cells were determined by substituting blood with complete medium or 4% HCl, respectively. Plates were centrifuged at 500 × *g* for 10 min to create a buffy coat layer of leukocytes and target cells on top of the RBCs before a 4-h incubation period. Following incubation, plates were again centrifuged, and aliquots of 100 μl of the supernatant were recovered from each well for assessment of radioactivity in a gamma counter. SP and MAX release of radioactivity from tumor cells were measured separately for each of the six tumor concentrations, and the percent specific lysis was calculated for each E:T cell ratio, using the formula: $(0.8 \times X - SP)/(MAX - SP) \times 100$, where X is the experimental release. The X value is multiplied by 0.8 to correct for the reduction in the supernatant volume into which ^{51}Cr is released, as caused by the presence of RBCs in the wells (MAX and SP are assessed in the absence of RBCs).

[3] Abbreviations used in this paper: MP, metaproterenol; PMNs, polymorphonuclear cells; SP, spontaneous release; MAX, maximal release; WBC, white blood cell; BBB, blood-brain barrier.

Table I. *Numbers and percentage of NK and other WBCs at different times after injection of 1 mg/kg of metaproterenol (MP) or saline*[a]

Time post injection	NK/μl	NK % of WBCs	WBCs/μl	PMNs/μl	PBMCs/μl
Baseline	349.0 ± 36.4	3.91 ± 0.35	9083.7 ± 533.0	1805.4 ± 128.1	6929.2 ± 442.5
MP 10 min	692.9 ± 116.9**	6.31 ± 0.60**	10642.0 ± 1159.2	2457.7 ± 257.6	7491.4 ± 943.1
MP 20 min	619.6 ± 117.4**	6.37 ± 0.61**	9323.4 ± 1026.1	2329.3 ± 229.8	6374.6 ± 816.1
MP 40 min	452.2 ± 59.6	6.00 ± 0.57**	7466.5 ± 699.5	2207.7 ± 282.8	4806.5 ± 533.4**
MP 1 h	343.6 ± 39.3	6.61 ± 1.13**	5819.6 ± 565.0**	1673.6 ± 222.3	3802.2 ± 454.7**
MP 3 h	278.8 ± 46.7	2.89 ± 0.32	9683.0 ± 865.4	3457.6 ± 530.9**	5946.6 ± 558.3
MP 5 h	318.9 ± 49.7	3.20 ± 0.41	9792.3 ± 687.4	2663.8 ± 342.7*	6809.5 ± 438.6
MP 18 h	385.6 ± 78.3	3.72 ± 0.47	9747.7 ± 790.7	1772.9 ± 258.7	7589.0 ± 694.6
Saline 10 min	252.1 ± 32.1	3.04 ± 0.32	8323.1 ± 579.5	2540.7 ± 334.0	5530.3 ± 434.6
Saline 20 min	315.1 ± 33.7	3.32 ± 0.36	9623.0 ± 618.5	2003.3 ± 228.4	7304.5 ± 561.8
Saline 40 min	279.8 ± 60.1	3.08 ± 0.37	8560.3 ± 1245.2	2525.0 ± 663.5	5755.5 ± 697.0

[a] Values are means ± SEM. Significant changes from baseline levels are indicated by *$p < 0.05$ and **$p < 0.01$.

MADB106 tumor cells

MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma (MADB100) chemically induced in the inbred F344 rat (38). The MADB106 tumor metastasizes only to the lungs, and the number of tumor cells retained in the lung 24 h following i.v. inoculation as well as the consequent metastases enumerated weeks later are highly dependent on NK activity (38, 39). The cell line was maintained in 5% CO_2 at 37°C in monolayer cultures in complete medium and was separated from the flask using 0.25% trypsin.

Induction and counting of tumor metastases (in vivo)

Rats were lightly anesthetized with halothane, and 10^5 MADB106 tumor cells were injected into their tail vein in 0.5 ml of PBS. Three weeks later, rats were euthanized, and their lungs were removed and placed for 24 h in Bouin's solution (72% saturated picric acid solution, 23% formaldehyde (37% solution), and 5% glacial acetic acid). After being washed in ethanol, visible surface metastases were counted by a researcher ignorant of the origin of each lung.

Radiolabeling of tumor cells and assessment of lung tumor retention

For assessment of lung tumor retention, DNA radiolabeling of tumor cells was accomplished by adding 0.4 μCi/ml of ^{125}Iododeoxyuridine (Rotem Taasiot, Dimona, Israel) to the growing cell culture 1 day before harvesting the cells for injection.

For tumor cell injection, rats were lightly anesthetized with halothane and had 4×10^5/kg ^{125}iododeoxyuridine-labeled MADB106 tumor cells in approximately 0.5 ml of PBS injected into their tail vein. Twenty-four hours later, rats were euthanized with halothane, and their lungs removed and placed in a gamma counter for assessment of radioactive content. The percentage of tumor retention was calculated as the ratio between radioactivity measured in the lungs and total radioactivity in the injected tumor cell suspension. Our previous studies have indicated that the levels of lung radioactivity reflect the numbers of viable tumor cells in the lungs (for more information, see Ref. 39).

Selective in vivo depletion of NK cells

Two days before tumor inoculation, approximately 1.5 mg/kg of anti-NKR-P1 was injected i.v. under light halothane anesthesia. In a previous study using the above-mentioned dose of anti-NKR-P1, we showed a complete abolition of blood and splenic NK cytotoxicity and a 100-fold increase in the lung retention and metastatic colonization of MADB106 tumor cells (39). In addition, we have used other mAbs (R73, W3/25, and ED2), mouse serum, and saline as controls for the administration of anti-NKR-P1 and have found them to have no effect (39).

Statistical analysis

For statistical analysis, analysis of variance was conducted, and, provided significant group differences existed, planned contrasts were used. The p levels indicate planned comparisons between each experimental group and the relevant control group. Linear trend analysis was conducted to identify dose dependency. Repeated measures analysis of variance (for E:T cell ratios) was used to compare percent specific killing between different groups in Expt. 2. In Expt. 3, lytic units were calculated using the formula $100/ET_{50}$, where ET_{50} is the E:T cell ratio needed to lyse 50% of target cells. The regression exponential fit method (48) was used to infer ET_{50} from the data.

Results

Expt. 1: time course of changes in blood number of NK cells and other white blood cells (WBC) following MP administration

FACS analysis was used to record the number of NK cells per microliter of blood and their percentage within the total WBCs at different times after treatment with the nonspecific β-agonist MP. Changes in the number of total WBCs, PBMCs, and PMNs were also monitored.

Eleven groups of rats were used: seven groups were injected s.c. with 1 mg/kg of MP at different times before blood withdrawal (10 min, 20 min, 40 min, 1 h, 3 h, 5 h, and 18 h), three groups were injected with saline (10, 20, and 40 min before blood withdrawal), and one group was not injected at all (baseline).

The findings are summarized in Table I. The number of NK cells per microliter of blood doubled within 10 min ($p < 0.0001$), declined to the baseline level within 1 h, and remained fairly constant at the baseline level (~300 cells/μl) thereafter (Fig. 2). The number of PBMCs decreased by 50% at 1 h following treatment ($p < 0.0001$), and the number of PMNs increased by 90% at 3 h following treatment ($p < 0.0001$). Although the number of NK cells per microliter of blood had already returned to baseline by 1 h, the

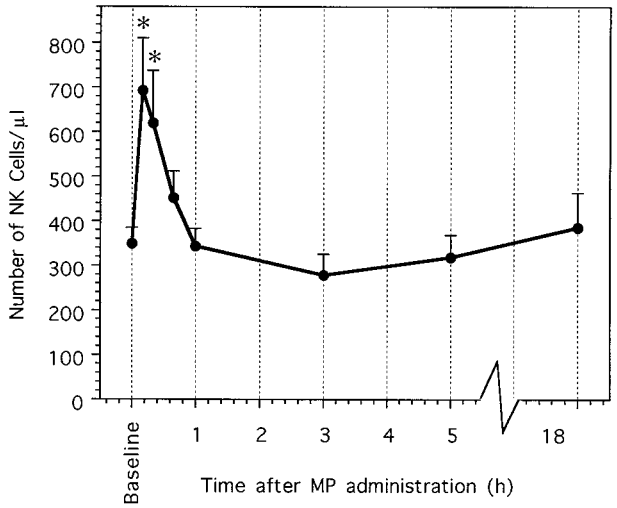

FIGURE 2. Mean numbers (±SEM) of NK cells per microliter of blood at 10 min, 20 min, 40 min, 1 h, 3 h, 5 h, and 18 h after s.c. injection of 1 mg/kg of MP. Time 0 refers to baseline NK numbers (untreated animals). The asterisk indicates a significant deviation ($\alpha < 0.01$) from the baseline.

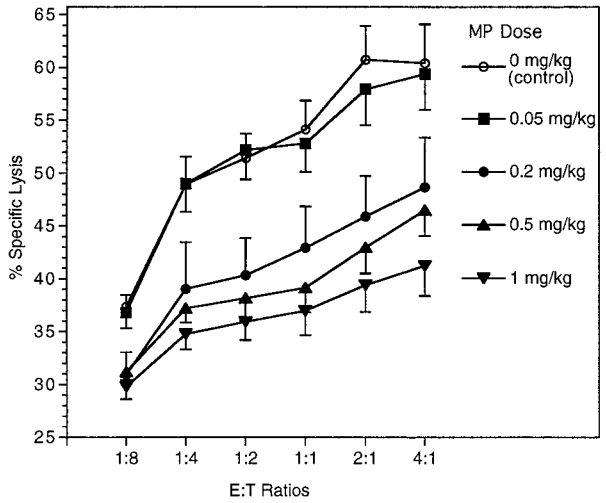

FIGURE 3. Mean blood NK activity (±SEM) of rats treated with different doses of MP 1 h before blood withdrawal. E:T cell ratios refer to mean numbers of NK cells per target cells (YAC-1). A significant dose-dependent decrease in NK activity was evident.

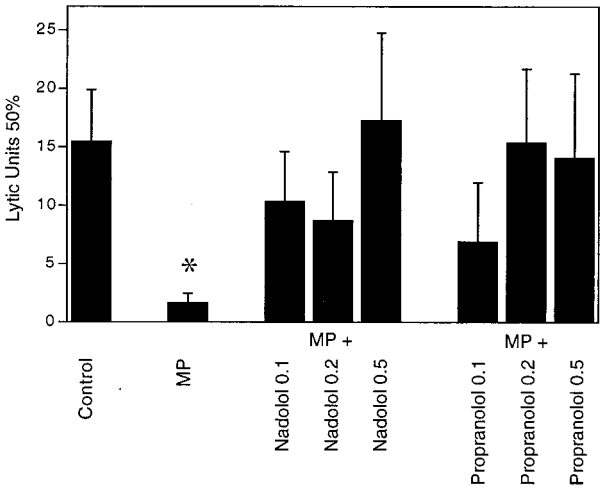

FIGURE 4. Blood NK activity of rats treated with 0.5 mg/kg of MP and different doses of the β-blockers, nadolol and propranolol. NK activity is expressed as mean lytic units at 50% target cell killing (±SEM). The asterisk indicates a significant deviation ($\alpha < 0.03$) from the control value.

decrease in the number of PBMCs at this time introduced a significant increase in the percentage of NK cells ($p < 0.0001$). The stress associated with saline injection did not affect cell numbers significantly.

Expt. 2: ex vivo effects of MP on blood NK cell cytotoxicity

Rats were divided into five groups, and each was given a different dose of MP (0, 0.05, 0.2, 0.5, and 1 mg/kg). Blood NK activity was assessed 1 h following MP administration when NK cell numbers were expected to return to the baseline level according to the results of Expt. 1. The number of blood NK cells was assessed for verification.

MP caused a dose-dependent decrease in NK activity (Fig. 3). Trend analysis using E:T cell ratios as repeated measures indicated a significant linear dose dependency ($p < 0.0001$). NK activity in the three highest doses of MP (0.2, 0.5, and 1 mg/kg) was significantly lower than that in controls ($p < 0.005$, $p < 0.001$, and $p < 0.001$, respectively). MP administration did not change the numbers of NK cells per microliters of blood in the samples tested for NK activity.

Expt. 3: peripheral vs central blockade of the ex vivo effect of MP

Unlike peripherally released adrenaline and noradrenaline, MP can cross the blood-brain barrier (BBB) to some extent (40). The effects of MP on NK activity can thus be related to the activation of central adrenergic circuits that modulate immunity. We therefore compared the efficiency of peripheral β-blockade to central β-blockade of MP-induced NK suppression.

Two β-blockers were used. The first, propranolol, is lipophilic and readily crosses the BBB. The second, nadolol, is hydrophilic and is confined to the periphery following s.c. administration (42). We verified that these two antagonists are equipotent in their peripheral effects; i.e., they are equally efficient in blocking the tachycardia caused by 0.5 mg/kg of MP (data not shown). Therefore, if propranolol is more efficient in blocking the effects of MP, a central mechanism would be implicated. The lowest doses of the two antagonists used were the minimum amounts required for blocking the peripheral effects of MP.

Animals were divided into eight groups: six were injected with MP (0.5 mg/kg s.c.) together with either nadolol or propranolol at doses of 0.1, 0.2, and 0.5 mg/kg s.c. (15 min before MP), one group was injected with MP alone, and one group received no drug.

MP markedly suppressed NK activity ($p < 0.03$; Fig. 4). The lowest doses of both nadolol and propranolol attenuated this suppression, and the highest doses completely abolished it. All doses of the β-blocker were sufficient for rendering the effect of MP statistically insignificant. No distinguishable difference in the efficacy of the two β-blockers in reversing the effect of MP was observed.

Expt. 4: in vivo effects of MP and nadolol on number of MADB106 metastases

To investigate the effects of β-adrenergic stimulation on the metastatic spread of an NK-sensitive tumor, MP and the β-adrenergic antagonist, nadolol, were employed.

Rats were divided into six equal groups using a 2 × 3 design: three groups received nadolol (0.2 mg/kg s.c.), while the other three groups served as control groups. Fifteen minutes later, the nadolol and control groups were injected s.c. with saline containing 0, 1, or 3 mg/kg of MP. One hour later, all animals were inoculated with MADB106 tumor cells. Lung metastases were counted 3 wk later.

In control animals, MP (1 and 3 mg/kg) significantly increased the number of metastases by 5.5 and 7.5 times, respectively ($p < 0.02$ and $p < 0.005$; Fig. 5). In nadolol-treated animals the effects of MP were not significant. The number of metastases in animals treated with either 1 or 3 mg/kg of MP was significantly smaller in nadolol-treated animals than in controls ($p < 0.05$ and $p < 0.02$, respectively).

Expt. 5: in vivo effects of MP on lung tumor retention

A complementary index of host resistance to tumor metastasis is the retention of MADB106 tumor cells in the lungs 24 h following inoculation. This index is highly predictive of the actual number of metastases that would have otherwise arisen weeks later (39). Additionally, because the metastatic process of MADB106 is sensitive to NK activity only in the first 24 h following inoculation, lung

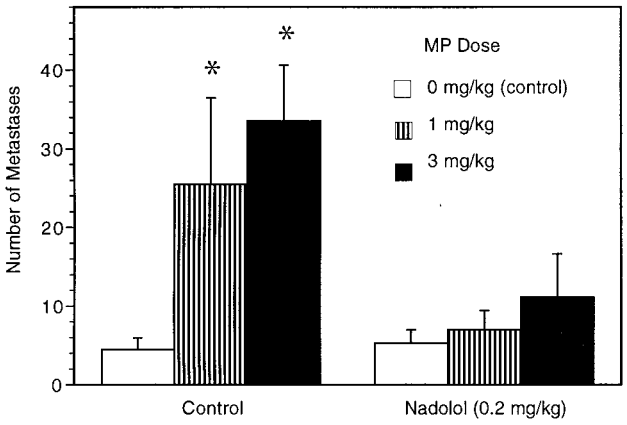

FIGURE 5. Mean numbers (±SEM) of lung MADB106 metastases in rats treated with different doses of MP and the β-antagonist nadolol. Nadolol significantly reduced the metastasis-enhancing effect of MP. The asterisk indicates a significant deviation ($\alpha < 0.02$) from the control value.

tumor retention is less affected by factors unrelated to NK activity than is the number of metastases.

Rats were divided into eight groups: six groups were treated 1 h before tumor inoculation with increasing doses of MP (0, 0.3, 0.6, 0.8, 1, 3, and 9 mg/kg s.c.), and the two remaining groups were injected with either vehicle or MP (0.8 mg/kg s.c.) simultaneously with tumor inoculation.

MP elevated lung tumor retention in a dose-dependent manner ($p < 0.0001$), reaching a 13-fold increase (Fig. 6). Because of a much higher variance within the group treated with 9 mg/kg of MP (violating statistical assumptions), analysis of variance was conducted only on the seven lower doses. Planned contrasts revealed that, with the exception of the lowest dose (0.3 mg/kg), MP-treated rats exhibited significantly higher retention than controls ($p < 0.005$). MP elevated tumor retention to a similar extent when administered simultaneously with tumor inoculation and when administered 1 h previously.

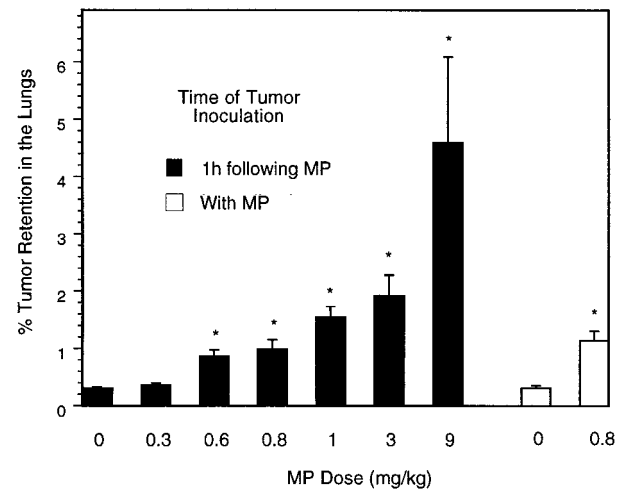

FIGURE 6. The effect of increasing doses of MP on the percentage of radiolabeled MADB106 tumor cells retained in the lungs 24 h after tumor inoculation (mean ± SEM). Tumor was inoculated either 1 h or immediately following MP treatment. A significant dose-dependent increase in lung tumor retention was evident. Tumor retention was similarly affected with both times of inoculation. The asterisk indicates a significant deviation ($\alpha < 0.005$) from the control value (0 mg/kg of MP).

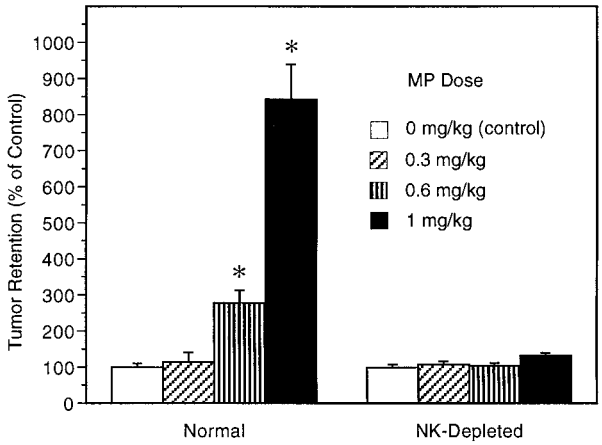

FIGURE 7. The effect of increasing doses of MP on lung retention of radiolabeled MADB106 tumor cells in NK-depleted or normal rats. Values are expressed as a percentage of the relevant control value (±SEM). The absolute level of lung tumor retention in the control groups was 223 times higher in NK-depleted rats than in normal rats (not shown). The asterisk indicates a significant deviation ($\alpha < 0.0001$) from the control value (100%).

Expt. 6: the in vivo effects of MP in NK-depleted rats

Although the MADB106 tumor model is known to be highly sensitive to NK activity (38), other factors may also mediate the effects of β-adrenergic stimulation on host resistance to this tumor (see *Discussion*). To assess the relative contributions of such factors, we compared the effects of MP in normal rats to its effects in rats depleted of NK cells. Normal and anti-NKR-P1-treated rats were divided into four subgroups, each receiving a different dose of MP (0, 0.3, 0.6, and 1 mg/kg s.c.). One hour later, rats were inoculated with MADB106 tumor cells, and after 24 h, lungs were removed to assess tumor retention.

NK depletion in itself increased tumor retention by 223 times. When tumor retention was expressed as a percentage of the retention levels of control groups (not treated with MP), there was a significant interaction between the effects of MP and depletion of NK cells ($p < 0.0001$; Fig. 7). In normal rats, MP caused a significant increase in tumor retention: 0.3 mg/kg of MP had no significant effect, 0.6 increased retention by threefold ($p < 0.0001$), and 1 mg/kg of MP increased retention by eightfold ($p < 0.0001$). In NK-depleted rats, however, there was no significant increase in any of the doses used, although the highest MP dose induced a 30% increase in tumor retention.

Discussion

Our findings support the assertion that peripheral β-adrenergic activation temporarily increases the number of circulating NK cells, but suppresses NK activity for a prolonged period, consequently compromising host resistance to metastasis in the rat. Specifically, 1 mg/kg of MP, a β-adrenergic agonist, rapidly doubled the numbers of blood NK cells, an effect that receded within 1 h following MP administration. At that time, blood NK activity was shown to be suppressed by MP in a dose-dependent manner. The β-antagonists, propranolol, which crosses the BBB, and nadolol, which does not, both attenuated this effect of MP. To study the biologic relevance of these adrenergic effects, animals were challenged with the MADB106 adenocarcinoma, an NK-sensitive tumor line that metastasizes only to the lungs. The host resistance to metastasis of this tumor was compromised by MP; the retention of tumor cells in the lungs increased, as did the number of metastases

counted 3 wk later. These effects were also attenuated by β-adrenergic blockade. Finally, MP was ineffective in increasing tumor retention in the lungs of NK-depleted animals. This further implicates NK cells in the mediation of the tumor-enhancing effects of MP.

The physiologic relevance of adrenergic activation by MP

The doses of MP used in this study are within the physiologically relevant range. We and others have found that a dose of 0.1 mg/kg was the minimum needed to induce tachycardia (41). Furthermore, in the present study the threshold doses of MP required to suppress NK activity and tumor resistance were approximately the same as those needed to induce tachycardia.

Unlike peripherally released catecholamines, MP can cross the BBB to some extent (40). Thus, it might be suggested that central, rather than peripheral, mechanisms underlie its immunomodulatory effects. However, our findings provide evidence to the contrary. First, both propranolol and its equipotent hydrophilic analogue, nadolol, which does not cross the BBB (42), blocked the NK-suppressive effects of MP with the same efficacy. Second, nadolol also blocked the tumor-enhancing effect of MP. Finally, our recent studies employing the administration of adrenaline, the endogenous mixed adrenergic agonist, replicated many of the effects of MP reported here (36). It therefore appears that adrenergic NK suppression is mediated peripherally and is not idiosyncratic to MP's longer duration of effect or to its selective β-agonism.

Role of NK cells in mediating the tumor-enhancing effects of MP

The MADB106 tumor model was used to elucidate the biologic significance of β-adrenergic alterations in NK activity. To this end, it was essential to determine whether MP compromises the host resistance to MADB106 metastasis through the suppression of NK activity or through other adrenergic effects (e.g., tachycardia, hypertension, vasodilatation, and elevated capillary permeability).

The involvement of NK cells in mediating the tumor-enhancing effects of β-adrenergic activation is supported by the following. 1) The MADB106 tumor meets the prerequisite of being sensitive to NK activity in vivo (38, 39). This sensitivity was confirmed in the current study when selective NK depletion increased tumor retention by >200 times. In previous studies, a correlation between NK activity and MADB106 tumor clearance was observed under various experimental conditions (8, 49–51). 2) MP suppressed NK activity and enhanced tumor metastasis; the two effects were characterized by similar patterns of dose dependency. 3) Direct evidence for NK mediation is derived from the ineffectiveness of MP in suppressing tumor resistance in rats selectively depleted of NK cells. This finding indicates that in the absence of NK cells, the various physiologic effects of MP do not enhance tumor metastasis. Our previous studies suggest that this lack of effect cannot be attributed to a ceiling effect induced by the high levels of tumor retention in NK-depleted rats, as some treatments (e.g., surgery) can enhance tumor retention in NK-depleted animals as effectively as in normal rats (47). Taken together, these observations strongly suggest that the suppression of NK cells underlies the tumor-enhancing effects of MP, thereby demonstrating the biologic significance of adrenergic modulation of NK activity.

A common mechanism for NK cell deactivation and redistribution?

In vitro studies have suggested that both detachment of NK cells from the epithelium and suppression of NK cytotoxicity are induced by the activation of β_2 receptors expressed by NK cells (13, 23). These two responses may share a common cellular mechanism: a reduction in adherence to cells (either epithelial or cancerous). In our study, however, these two effects differed in their time courses. Whereas the elevated number of NK cells in the blood had receded by 1 h following MP administration, the suppression of both NK activity and NK-related tumor resistance was pronounced at this time. The rapid return to the baseline concentration of blood NK cells seems surprising in view of the slower clearance rate of MP ($t_{1/2} = \sim 2$ h) (40, 41). It is possible that following MP administration NK cells only pass through the blood on their way to other immune compartments, where they will stay throughout the full span of β-adrenergic stimulation. This passage ends quickly, whereas the new pattern of distribution persists. Alternatively, the cellular process directing redistribution may be self-terminating.

Relations to studies in humans

Findings from human studies have been inconsistent. Although a number of in vitro studies have demonstrated NK suppression by adrenaline (11–13), ex vivo experiments, in contrast to our findings in rats, have usually reported augmentation of NK activity following infusion of adrenaline (14–16, 27).

Although species differences are possible, it is our hypothesis that the main factor distinguishing our study from most studies in humans is the control over the number of NK cells tested for cytotoxicity. The majority of studies administering adrenaline or stressful stimuli in humans drew blood either during the experimental manipulation or immediately following it, when the percentage of blood NK cells is now known to be markedly elevated (up to 600%) (20–22). Cytotoxicity was assessed per PBMC, rather than per NK cell. Thus, it could be suggested that the increased NK activity reported in these studies reflects an increased number of NK cells.

A number of studies have retroactively compensated for the increase in the percentage of NK cells, but have still reported inconsistent findings (17, 21, 25, 27), probably due to differences in the methods used to enumerate NK cells and compensate for changes in their number. In the current study, blood was drawn 1 h after adrenergic activation when the numbers of blood NK cells had returned to baseline levels. Our findings, which indicate a marked decline in both NK activity per cell and tumor resistance, may thus reflect the long term response to adrenergic activation, a response independent of NK cell redistribution. In agreement with this interpretation, studies in humans have demonstrated that 1 h after adrenaline infusion (26, 28) or exposure to stress (17), NK activity was suppressed. An additional study indicated that a decrease in NK activity per cell is already evident during the rise in blood NK cell numbers (52).

Implications regarding host resistance to metastasis

It appears that the adrenergic effect on NK cells consists of two components: first, a large transitory increase in NK cell number leading to an increase in NK activity per milliliter of blood (and per PBMC), and second, a prolonged suppression of NK activity related to changes in individual cell cytotoxicity. If this is indeed the case, the following considerations suggest that the overall effect of acutely elevated levels of catecholamines is detrimental to NK-mediated resistance to cancer. First, a rapid rise in the number of circulating blood NK cells results from redistribution and is therefore accompanied by a decrease in NK cell numbers in other immune compartments, which may be as important for host resistance to cancer. Second, the changes in circulating NK cell number are transitory, lasting 20 to 50 min, whereas metastatic risk to the organism endures for much longer. The current study, using a tumor model sensitive to NK activity throughout the 24 h following

inoculation (38, 39), suggests that the cumulative effects of adrenergic stimulation on NK activity are suppressive. Moreover, the increased number of circulating NK cells during the first hour after MP injection did not improve host resistance to MADB106. The tumor-enhancing effects of MP were no smaller when tumor cells were injected simultaneously with MP than when they were injected 1 h later, when the number of NK cells had returned to baseline. Taken together, these considerations suggest that, overall, acutely elevated levels of catecholamines reduce host resistance to metastasis.

Although it is not our suggestion that everyday stress seriously jeopardizes one's health, we do believe that our findings have implications when a high risk of metastatic spread is combined with elevated sympathetic activity. A widespread situation in which these conditions are met is surgical removal of a tumor in patients with positive lymph nodes. It has been shown that surgeries and their associated stress involve major sympathetic activity and are often followed by distinct suppression of NK activity (53–55). Transitory dysfunction of NK cells may create a window of opportunity for metastases to be established. This may be the result either of a release from control over dormant micrometastases or of an inability to destroy tumor cells released perioperatively. As surgical intervention is usually imperative, it is important to develop prophylactic measures to reduce such unnecessary risk. To this end, a more detailed understanding of the cellular mechanisms involved in the in vivo suppression of NK activity by catecholamines is desirable.

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